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Physiological levels of nitrate support anoxic growth by denitrification of *Pseudomonas aeruginosa* at growth rates reported in cystic fibrosis lungs and sputum

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Chronic *Pseudomonas aeruginosa* lung infection is the most severe complication in patients with cystic fibrosis (CF). The infection is characterized by the formation of biofilm surrounded by numerous polymorphonuclear leukocytes (PMNs) and strong O₂ depletion in the endobronchial mucus. We have reported that O₂ is mainly consumed by the activated PMNs, while O₂ consumption by aerobic respiration is diminutive and nitrous oxide (N₂O) is produced in infected CF sputum. This suggests that the reported growth rates of *P. aeruginosa* in lungs and sputum may result from anaerobic respiration using denitrification. The growth rate of *P. aeruginosa* achieved by denitrification at physiological levels (~400 μM) of nitrate (NO₃⁻) is however, not known. Therefore, we have measured growth rates of anoxic cultures of PAO1 and clinical isolates (*n* = 12) in LB media supplemented with NO₃⁻ and found a significant increase of growth when supplementing PAO1 and clinical isolates with ≥150 μM NO₃⁻ and 100 μM NO₃⁻, respectively. An essential contribution to growth by denitrification was demonstrated by the inability to establish a significantly increased growth rate by a denitrification deficient $\Delta nirS-N$ mutant at <1 mM of NO₃⁻. Activation of denitrification could be achieved by supplementation with as little as 62.5 μM of NO₃⁻ according to the significant production of N₂O by the nitrous oxide reductase deficient $\Delta nosZ$ mutant. Studies of the promoter activity, gene transcripts, and enzyme activity of the four N-oxide reductases in PAO1 (Nar, Nir, Nor, Nos) further verified the engagement of denitrification, showing a transient increase in activation and expression and rapid consumption of NO₃⁻ followed by a transient increase of NO₂⁻. Growth rates obtained by denitrification in this study were comparable to our reported growth rates in the majority of *P. aeruginosa* cells in CF lungs and sputum. Thus, we have demonstrated that denitrification is required for *P. aeruginosa* growth in infected endobronchial CF mucus.

Keywords: *Pseudomonas aeruginosa*, denitrification, growth, cystic fibrosis

INTRODUCTION

Chronic lung infection by *Pseudomonas aeruginosa* is the most severe complication in cystic fibrosis (CF) patients (Koch and Høiby, 2000). The infection is characterized by biofilm aggregates of *P. aeruginosa* within the endobronchial mucus surrounded by high densities of active polymorphonuclear leukocytes (PMNs) (Bjarnsholt et al., 2009). The activity of PMNs has been shown to be the major cause of O₂ depletion due to production of the reactive oxygen species superoxide (O₂⁻) and nitric oxide (NO) within sputum samples (Kolpen et al., 2010, 2014a). Combined with the O₂ consumption by lung epithelial cells, micro-oxic to anoxic conditions can develop in the airways of CF patients (Worlitzsch et al., 2002), which have been further evidenced by

the upregulation of the O₂ sensing anaerobic global regulator (Anr) in *P. aeruginosa* isolates (Hoboth et al., 2009) and the presence of obligate anaerobes such as *Prevotella* within sputum (Tunney et al., 2008). While O₂ depletion by microbial aerobic respiration is thus diminutive, *P. aeruginosa* has been shown to be actively growing within the mucus of lungs (Kragh et al., 2014) and sputum (Yang et al., 2008). As a facultative anaerobe, this growth may be attributable to *P. aeruginosa*'s ability to perform anaerobic respiration by denitrification.

Denitrification was first described as the liberation of gaseous nitrogen oxides (N-oxides) by bacteria (Gayon and Dupetit, 1886) and involves the utilization of N-oxides as alternative electron acceptors for respiration (Figure 1). This definition of

denitrification means that while nitrate (NO_3^-) reduction to nitrite (NO_2^-) contributes directly to energy generation via the proton pumping action of the membrane-bound nitrate reductase (NarGHIJ) (Chen and Strous, 2013), the first step in strict denitrification is actually NO_2^- reduction, where NO is the first gaseous product arising from NO_3^- reduction (Zumft, 2005).

Engagement of denitrification in infected CF lungs is in accordance with the production of the denitrification intermediate nitrous oxide (N_2O) in sputum (Kolpen et al., 2014b),

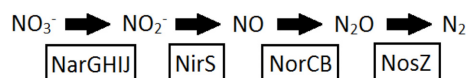


FIGURE 1 | Denitrification pathway. Reduction of nitrate to nitrite, nitric oxide, nitrous oxide, and finally dinitrogen. The four reductase enzymes nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reduction are boxed below. Nitrite reduction marks the first step of strict denitrification.

the presence of the denitrification marker OprF and antibodies against OprF in lung secretions and sera (Yoon et al., 2002), antibodies against Nar in sera (Beckmann et al., 2005) and the upregulation of the denitrification reductases in CF isolates (Son et al., 2007; Hoboth et al., 2009; Lee et al., 2011). Additionally, after antimicrobial treatment the infected sputum content of NO_3^- increases (Grasemann et al., 1998) indicating a reduction in the activity of denitrifying cells.

MATERIALS AND METHODS

BACTERIAL STRAINS AND PLASMIDS

The strains and plasmids used in this study are listed in **Table 1**. Primers used in this study are listed in **Table 2**. The wild type strain used for experiments and reporter plasmid insertion was PAO1 obtained from the Pseudomonas Genetic Stock Center (www.pseudomonas.med.ecu.edu, strain PAO0001). The Holloway PAO1 wild type was used as the control for the examinations of the Holloway PAO1 $\Delta nirS$ -N and the Holloway PAO1 $\Delta nosZ$ mutants. The PAO1 strains grew at similar rates under anoxic conditions.

Table 1 | *P. aeruginosa* strains used for this study.

Strains and plasmids	Details	Study
PLASMIDS		
pMEXGFP	pMEX9 derived promoter-probe vector; <i>egfp</i> Gm^r	Toyofuku et al., 2013
pMEXdsREDex	pMEX9 derived promoter-probe vector; <i>dsredexpress</i> Gm^r	This study
pMNarK ₁ -GFP	<i>narK₁</i> promoter region fused to <i>egfp</i> in pMEXGFP	This study
pMNirS-dsREDex	<i>nirS</i> promoter region fused to <i>dsredexpress</i> in pMEXdsREDex	This study
pMNorC-GFP	<i>norC</i> promoter region fused to <i>egfp</i> in pMEXGFP	This study
pMNosR-dsREDex	<i>nosR</i> promoter region fused to <i>dsredexpress</i> in pMEXdsREDex	This study
pET15b	Expression vector Ap^r	Novagen, Madison, WI, USA
pG19 <i>nir</i>	<i>nirS</i> to <i>nirN</i> deletion cassette in PG19II	Toyofuku et al., 2013
pG19 <i>nos</i>	<i>nosZ</i> deletion cassette in PG19II	This study
STRAINS		
<i>E. coli</i>		
S17-1	Mobilizer strain	Simon et al., 1986
<i>P. aeruginosa</i>		
PAO1 MH340	WT	Pseudomonas Genetic Stock Center
PAO1 <i>egfp</i>	Background control	Toyofuku et al., 2013
PAO1 <i>dsredexpress</i>	Background control	This study
PAO1 <i>narK₁-eGFP</i>	Nitrate reductase reporter	This study
PAO1 <i>nirS-dsredexpress</i>	Nitrite reductase reporter	This study
PAO1 <i>norC-eGFP</i>	Nitric oxide reductase reporter	This study
PAO1 <i>nosR-dsredexpress</i>	Nitrous oxide reductase reporter	This study
Holloway PAO1	WT	Holloway et al., 1979
Holloway $\Delta nirS$ -N	<i>nirS</i> to <i>nirN</i> deleted mutant	Toyofuku et al., 2013
Holloway $\Delta nosZ$	<i>nosZ</i> deleted mutant	This study
I M/NM	Clinical isolate	Kolpen et al., 2014c
II M/NM	Clinical isolate	Kolpen et al., 2014c
III M/NM	Clinical isolate	Kolpen et al., 2014c
IV M/NM	Clinical isolate	Kolpen et al., 2014c
V M/NM	Clinical isolate	Kolpen et al., 2014c
VI NM	Clinical isolate	Kolpen et al., 2014c
VII NM	Clinical isolate	Kolpen et al., 2014c

Gm^r (Gentamicin resistant), Ap^r (Ampicillin resistant), Mucoïd (M), Non-mucoïd (NM), Wild type (WT).

Table 2 | Primer sequences used in this study.

Primers	Sequence	Study
REPORTER PLASMIDS		
Gfp F1	5' – ACGCCAAGCTTGCATGCCTG – 3'	Toyofuku et al., 2013
pET15b-kpn	5' – GGGGTACCACTTTAAGAAGGAGATATACCATGG – 3'	Toyofuku et al., 2013
dsred R1	5' – GGGCTCGAGCTACAGGAACAGGTGGTGGCGGC – 3'	This study
dsred R2	5' – CGAGCTCTACAGGAACAGGTGGTGGCGGC – 3'	This study
pNarK ₁ F	5' – GGAATTCCTGGCGTGGTTGATCACCGC – 3'	This study
pNarK ₁ R	5' – TTCAAGCTTAGGCCAGGCCGTAGATCGGG – 3'	This study
pNirS F	5' – CCCAAGCTTCTTCATGTCGTCTTGGCGTG – 3'	This study
pNirS R	5' – CCCAAGCTTGCCCTTGAGCAATACCGGCAG – 3'	This study
pNorC F	5' – CGGAATTCGCGAGCGCTTCATGGAGCTGG – 3'	This study
pNorC R	5' – GCCAAGCTTGCCGAAATAGATGTTCTTGCC – 3'	This study
pNosR F	5' – CGGGATCCCCTGGTACCGTTACCTGAAGGC – 3'	This study
pNosR R	5' – CCCAAGCTTGATCACCTGCAGGTTGATCGG – 3'	This study
MUTANTS		
Δ nirS-N F1	5' – CGGAATTCCTAGAGGGTGAAGAAAGTGTCTGTC – 3'	Toyofuku et al., 2013
Δ nirS-N R1	5' – CGGGATCCGACCGGAATCAAGATTGCGTTGCTTTGC – 3'	Toyofuku et al., 2013
Δ nirS-N F2	5' – CGGGATCCGGTCGAGACGCCATCAACGGATATCC – 3'	Toyofuku et al., 2013
Δ nirS-N R2	5' – CCGAAGCTTGCATGTTACCATGATGAACACGAGC – 3'	Toyofuku et al., 2013
Δ nosZ F1	5' – CGCAAGCTTGGGTCTGGTACCAGAAGATTTCACAGG – 3'	This study
Δ nosZ R1	5' – GACTAGTGTGCTCATCTCGTGTCTCCTCAAGGG – 3'	This study
Δ nosZ F2	5' – GACTAGTCCGCAACAGACCTCGTCGATCACC – 3'	This study
Δ nosZ R2	5' – CGCAAGCTTCGAGTAGGTGATGTAGTTCATCAGGATGCC – 3'	This study
qPCR		
narK ₁ F	5' – CTCCTCCTGCTTCATGTTGCT – 3'	This study
narK ₁ R	5' – TGCTGCTTGATCGCGTAGTG – 3'	This study
nirS F	5' – GGC GGCTCGTGTTTCAT – 3'	This study
nirS R	5' – TGAAGGTGGTGTGACGTAGAG – 3'	This study
norC F	5' – AAGGCATGGCCAGGAACAT – 3'	This study
norC R	5' – CCAGGAACAGCAGGATGAAGA – 3'	This study
nosZ F	5' – CGCTGCACACCACTTTTCG – 3'	This study
nosZ R	5' – CACCAACTGGCTGTGATGA – 3'	This study
rpoD F	5' – ACAAGATCCGCAAGGTACTGAAG – 3'	This study
rpoD R	5' – CGCCCAGGTGCGAATC – 3'	This study

Forward (F), Reverse (R).

Plasmids containing eGFP or DsRed-Express tagged reporter genes were derived from pME as pG19nir and pG19nos plasmids carrying deletion cassettes of *nirS* to *nirN* and *nosZ* were constructed with the same procedure described previously (Maseda et al., 2004). The pG19II-derived plasmids were transferred into Holloway PAO1 by conjugating with *E. coli* S17-1 (Simon et al., 1986) followed by homologous recombination described elsewhere (Maseda et al., 2004). The mutants were analyzed by polymerase chain reaction (PCR) (Toyofuku et al., 2013).

The eGFP-based promoter probe plasmid, pMEXGFP, was constructed as described previously (Toyofuku et al., 2013). The promoter regions of *narK₁* and *norC* were amplified with pNarK₁ F/pNarK₁ R or pNorC F/pNorC R primer pairs and cloned into pMEXeGFP for promoter activity assays. pMEXdsREdEx was constructed in the same way, with *dsredexpress* amplified using the primer pairs Gfp F1/dsred R1 (Table 2) and post subcloning, the primer pair pET15b-kpn/dsred R2 (Table 2) was used to amplify a RBS-fused DsRed-Express to clone into the pMEX9

plasmid (Toyofuku et al., 2007). The promoter regions of *nirS* and *nosR* were amplified with the primer pairs pNirS F/pNirS R or pNosR F/pNosR R (Table 2) and cloned into the pMEDsREdEx for promoter activity assays.

The reporter plasmid insertion was achieved by preparation of electro-competent cells as previously described (Choi et al., 2005) and subsequent electroporation at 25 μ F, 200 Ω , 2.5 kV with 1 μ l of plasmid DNA for 100 μ l of electro-competent cells. Transformed cells were selected for by plating with 30 ng ml⁻¹ gentamicin (Sigma-Aldrich, St. Louis, MO, USA) and frozen cultures were produced from a single colony.

Clinical strains (5 mucoid and 7 non-mucoid) were isolated from expectorated sputum of 7 chronically infected CF patients (Kolpen et al., 2014b).

ANOXIC GROWTH EXPERIMENTS

In this study, we first establish experimental evidence for the ability of physiological levels of NO₃⁻ to support anoxic growth of

P. aeruginosa PAO1 wild type and 12 clinical isolates at rates comparable to those in CF lungs and sputum. To discern that a significant portion of the attained growth was attributable to denitrification, a strain incapable of denitrification due to a mutation in the nitrite reductase (Nir) operon was studied.

All experiments requiring anoxic conditions were performed in an anoxic bench (Concept 400, Thermo Scientific, Waltham, MA, USA) with $O_2 < 0.02\%$ routinely monitored with an luminescent dissolved oxygen O_2 sensor (HQ40d multi, HACH Company, Loveland, CO, US). Media was sealed with parafilm (Bemis, Neenah, WI, USA) and allowed to equilibrate for a minimum of 3 days. Strains were plated from frozen cultures and single colonies were used to initiate normoxic overnight cultures. They were then adjusted to OD_{600} 0.1 and regrown to OD_{600} 0.4 to ensure all cells were in the exponential growth phase. The culture was inoculated by diluting 1:100 in relevant anoxic Luria-Bertani broth (LB) media supplemented with KNO_3 (Sigma-Aldrich) to obtain the noted concentrations of NO_3^- in 2 ml glass vials (Schuett Biotec, Göttingen, DE), which were sealed inside the anoxic bench before transfer to shaking at 200 rpm. Physiological levels of NO_3^- here are considered to be approximately $400 \mu M$ with a maximum of $1 mM$ based on the concentrations of NO_3^- detected in CF sputum (Grasemann et al., 1998; Palmer et al., 2007; Kolpen et al., 2014a). Vials were shown to remain anoxic for a minimum of 2 days using anaerobic indicator strips (SSI Diagnostica, Hillerød, Denmark) that changed from white to blue in the presence of oxygen. Optical density was measured directly in the vials at 600 nm (OD_{600}) by a spectrophotometer (Shimadzu, Kyoto, JP) and values obtained were shown to correlate linearly to dilutions of an overnight culture in LB at OD_{600} values > 0.02 . Accordingly, only OD values higher than 0.02 were used in growth rate calculations. Growth rates were calculated as described (Widdel, 2007).

The suitability of the assay was demonstrated in cultures of PAO1 supplemented with $100 mM NO_3^-$, where our measured growth rate of 0.7 divisions h^{-1} and final OD_{600} of 1.0 (data not shown) corresponded well with previously reported growth rates of *P. aeruginosa* (Alvarez-Ortega and Harwood, 2007; Lee et al., 2012; Fang et al., 2013).

PROMOTER ACTIVITY—REPORTER STRAINS

To further confirm the engagement of denitrification, the transcripts and promoter activity of the four N-oxide reductases (Nar, Nir, Nor, Nos) were studied using qRT-PCR and fluorescent reporter strains, respectively. Reporter strains with the promoter region of the four denitrification reductase operons; *narK₁K₂GHJ*, *nirSMCFDLGHJEN*, *norCBD*, *nosRZDFYL* (Schobert and Jahn, 2010) fused to fluorescent tags of either eGFP or DsRed-Express (Table 1) were used to study promoter activation under anoxic conditions with $400 \mu M KNO_3$ supplemented LB as described above.

GFP and DsRed require O_2 for chromophore maturation (Craggs, 2009; Strack et al., 2010). Consequently, $1600 ng ml^{-1}$ chloramphenicol (Sigma-Aldrich) was added to the cultures to prevent new protein synthesis (Toyofuku et al., 2013) with shaking for 1 h at $37^\circ C$ in atmospheric air prior to measurement to allow the chromophore to develop—termed oxalic fluorescence

recovery (OFR) (Zhang et al., 2005). The signal was detected by flow cytometry as described below. The fluorescence values obtained were corrected for background fluorescence seen in the control strains with *eGFP* and *dsredexpress* unconjugated to a promoter.

FLOW CYTOMETRY

Detection of reporter strain fluorescence was performed using a FACSCanto flowcytometer (BD Biosciences, San Jose, CA, USA) with a 488 nm argon laser. eGFP was detected in FL-1 with a 502 nm long pass filter and a 530/30 nm band pass emission filter. DsRed-Express fluorescence was collected in FL-2 with a 556 nm long pass filter and 585/42 nm band pass emission filter. To maximize resolution, samples were analyzed at low flow rate corresponding to $10 \mu l min^{-1}$. At least 10,000 events were recorded for each sample. Cytometer Setup and Tracking Beads (BD Biosciences) were used for instrument calibration, and flow data were processed and analyzed by Diva (BD Biosciences).

DENITRIFICATION GENE TRANSCRIPT EXPRESSION—RNA EXTRACTION AND REVERSE TRANSCRIPTION

PAO1 was grown in triplicate for 3 h under anoxia with either unsupplemented LB or LB with $400 \mu M KNO_3$. The 3 h time point was chosen as it represented the approximate peak of promoter activity, as seen in the reporter strains. After 3 h, the cultures were mixed with 2 volumes of RNeasy lysis buffer (Ambion, Austin, TX, USA) within the anoxic bench for transcript stabilization and kept at $4^\circ C$ overnight before freezing at $-80^\circ C$. The cultures were subsequently thawed on ice, centrifuged to harvest the cells and lysed with $100 \mu l$ of $1 mg ml^{-1}$ lysozyme (Affymetrix, Cleveland, OH, USA) at room temperature for 13 min. Total RNA was extracted with RNeasy Mini Kits (Qiagen Denmark, Limburg, NL) and contaminating chromosomal DNA was removed by treatment with RQ1 RNase-free DNase (Promega, Madison, WI, USA). RNA quality was detected in a NanoDrop spectrophotometer (Thermo Scientific). cDNA was synthesized from 250 to 500 ng of RNA (depending on the concentration extracted) and High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA, US) with a final reaction volume of $20 \mu l$ in a 2720 Thermal Cycler (Applied Biosystems). The conditions consisted of 5 min at $25^\circ C$, 30 min at $42^\circ C$, 5 min at $85^\circ C$ and subsequent holding at $4^\circ C$. cDNA was frozen at $-20^\circ C$. Negative controls contained template with the Master Mix replaced with water. cDNA was diluted 1:100 for qPCR.

PRIMER DESIGN

Primers for the denitrification genes *narK₁*, *nirS*, *norC*, *nosR* and the reference gene *rpoD* were designed using Integrated DNA Technologies Primer Quest software (<http://www.idtdna.com>) using sequences from the PAO1 Genome Sequence, *Pseudomonas* Genome Database (Winsor et al., 2011) (Table 2).

QUANTITATIVE PCR

Each PCR mix had a final volume of $20 \mu l$ containing $10 \mu l$ SYBR Green qPCR Master Mix 2x (Applied Biosystems) $2 \mu l$ cDNA, $2 \mu l$ of $2 pmol \mu l^{-1}$ forward and reverse primers and $4 \mu l$ water. Quantitative real-time PCR was performed with a StepOnePlus,

Real-Time PCR system (Applied Biosystems) with initial denaturation at 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 30 s.

Data were normalized to the expression of *rpoD*, a housekeeping gene encoding the primary sigma factor for RNA polymerase found to be constitutively expressed in *P. aeruginosa* (Savli et al., 2003). To control for variations between runs, the housekeeping and target genes for each of the three samples were amplified in one 96-well plate. Expression of the target genes in the cultures with 400 μM KNO_3 was compared to the unsupplemented cultures and the difference expressed as n-fold change.

NO_3^- AND NO_2^- QUANTIFICATION

For measuring NO_3^- and NO_2^- concentration in the culture media, the supernatant was harvested and sterile filtrated through a syringe filter (TRP, Trasadingen, SUI) (pore size 0.22 μm) and frozen at -20°C for later measurement. NO_3^- and NO_2^- were detected in 96-well microtiter plates using a modified Griess nitrite/nitrate colorimetric assay (Cayman Chemicals, Ann Arbor, MI, USA) according to manufacturer's instructions and as previously described (Kolpen et al., 2014a). Briefly, NO_2^- concentration was estimated by incubating with the Griess Reagent, converting NO_2^- into a purple azo-compound detected by absorbance at 540 nm in a Multiskan EX plate reader (Thermo Scientific). For NO_3^- concentration, NO_3^- was first converted to NO_2^- by incubation with NO_3^- reductase and subsequent addition of the Griess reagent to detect total NO_3^- plus NO_2^- . The NO_3^- concentration was calculated as the difference between the total NO_2^- and NO_3^- concentration and the NO_2^- concentration. The detection limit of the assay was $\sim 2 \mu\text{M}$ for NO_2^- and $\sim 5 \mu\text{M}$ for NO_3^- .

MICROSENSOR MEASUREMENTS OF N_2O

Additionally, reductase activity was elucidated by measuring consumption of NO_3^- and production of NO_2^- as well as measurements of N_2O gas accumulation in a ΔnosZ mutant strain incapable of the final reduction step in denitrification of N_2O to

N_2 . Each small glass vial (Schuett Biotec, Göttingen, DE) containing anoxic planktonic cultures of either PAO1 WT or the ΔnosZ mutant were placed in a 37°C heated rack and kept sealed prior to individual measurements, upon which they were shaken and the lid removed. Immediately after, N_2O was detected in the media using an amperometric N_2O microsensor (Andersen et al., 2001) with a tip diameter of 25 μm (Unisense A/S, Aarhus, DK) as previously described (Kolpen et al., 2014a). Each measurement consisted of a 5 s wait period followed by 5 s measurements taken at 7, 1 mm intervals with data acquisition and sensor positioning controlled by the Sensortrace Pro 2.0 (Unisense A/S) dedicated software. Mean experimental N_2O concentration was calculated from triplicates of three individual experiments. The final mean was corrected for the background signal detected in pure LB.

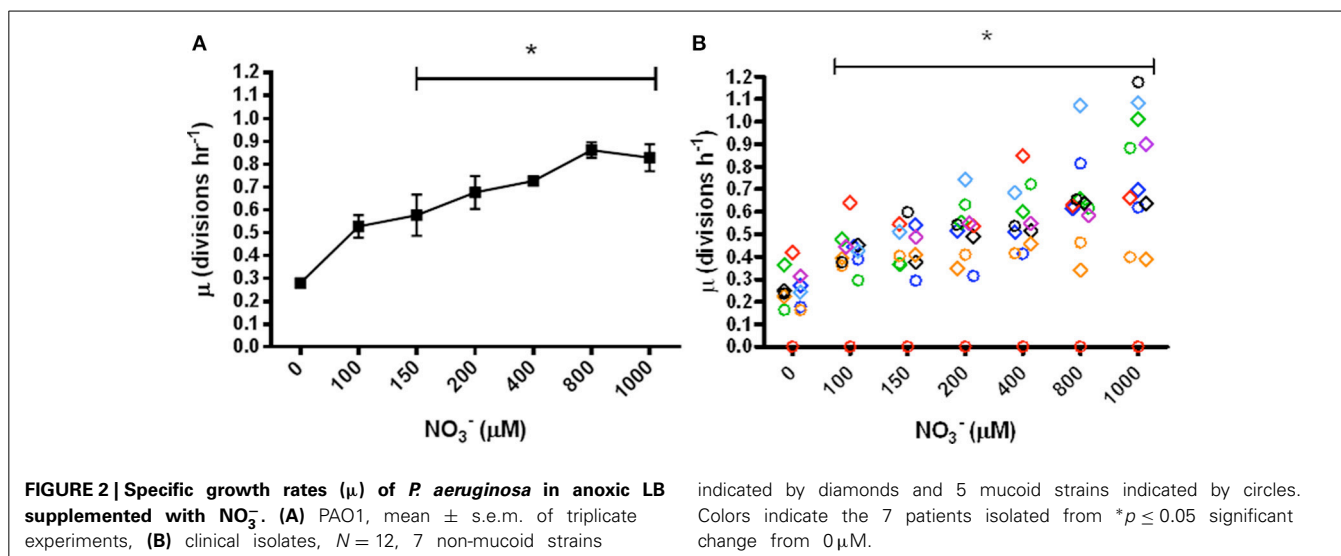
STATISTICAL ANALYSIS

Data was analyzed for statistical significance with Prism 4 software (GraphPad Software, Inc., La Jolla, CA, USA). Unless otherwise mentioned, parametric data was analyzed by One-Way ANOVA with Dunnett's multiple comparison test and non-parametric by Friedman's test with Dunn's multiple comparison. $P < 0.05$ was considered significant.

RESULTS

DENITRIFICATION HAS A SIGNIFICANT ROLE IN ANOXIC GROWTH OF PAO1 AND CLINICAL ISOLATES AT PHYSIOLOGICAL LEVELS OF NO_3^-

The specific growth rates of *P. aeruginosa* grown for 20 h in anoxic LB supplemented with physiologically relevant concentrations of NO_3^- (0–1 mM of NO_3^-) were calculated. PAO1 (Figure 2A) and 12 clinical isolates (5 mucoid and 7 non-mucoid) (Figure 2B) displayed a significant increase in specific growth rate as compared to the unsupplemented control at $\geq 150 \mu\text{M}$ NO_3^- ($p = 0.0138$) and $\geq 100 \mu\text{M}$ ($p = 0.0132$) NO_3^- , respectively. Despite the considerable variation between the growth rates of the clinical isolates, there was no significant correlation with the duration of infection as well as no significant difference between the growth rates of the mucoid and non-mucoid clinical isolates.



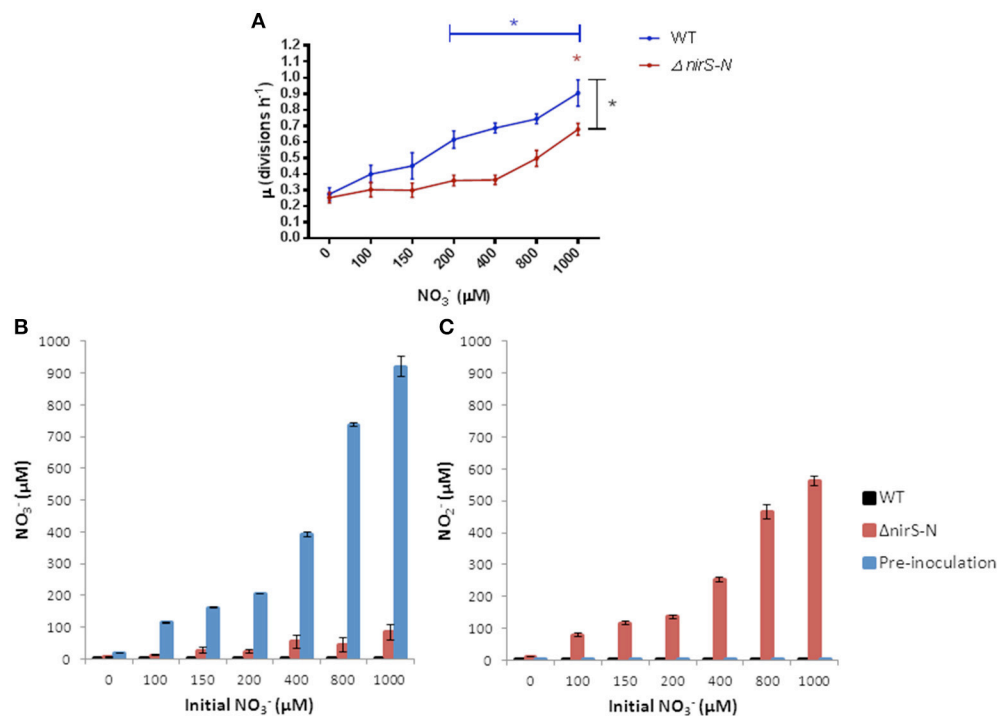


FIGURE 3 | (A) Specific growth rates (μ) of *P. aeruginosa* (WT and $\Delta nirS-N$ PAO1) grown in anoxic LB supplemented with NO_3^- . Mean \pm s.e.m. of triplicate experiments, * $p \leq 0.05$ Blue and red * indicate significant increase in μ from 0 μM calculated by

One-Way ANOVA. Black * indicate significant difference ($p = 0.0316$) between conditions ($\geq 400 \mu M$) as calculated by Two-Way ANOVA. **(B)** NO_3^- and **(C)** NO_2^- content of anoxic sterile filtered growth media after 20 h.

The suitability of the assay was demonstrated in a pilot study with anoxic cultures of PAO1 supplemented with 100 mM NO_3^- , where our measured growth rate of 0.7 divisions h^{-1} and final OD₆₀₀ of 1.0 (data not shown) corresponded well with previously reported growth rates of *P. aeruginosa* (Alvarez-Ortega and Harwood, 2007; Lee et al., 2012; Fang et al., 2013).

To discern the portion of growth attributable to denitrification, we compared the specific growth rates in anoxic cultures supplemented with NO_3^- of the WT to a $\Delta nirS-N$ mutant, which is able to reduce NO_3^- to NO_2^- , but with a blocked first step of strict denitrification due to the inability to reduce NO_2^- to NO, under the same growth conditions were estimated (Figure 3A). NO_3^- supports modest growth of the $\Delta nirS-N$ mutant with increasing NO_3^- supplementation in accordance with Nar's contribution to proton motive force generation (Chen and Strous, 2013), but with a significant reduction in growth as compared to the WT at $NO_3^- \geq 400 \mu M$ ($p = 0.0316$). One mM NO_3^- was required to initiate a significant increase in growth rate from the unsupplemented control in the $\Delta nirS-N$ mutant ($p = 0.0021$).

The consumption of NO_3^- and the accumulation of NO_2^- in the sterile filtered growth media of the $\Delta nirS-N$ mutant at 20 h confirmed that the ability to reduce NO_3^- to NO_2^- was present in the $\Delta nirS-N$ mutant, but that the mutant failed to further reduce NO_2^- as opposed to the total consumption of NO_2^- by the WT. Furthermore, we demonstrated very low concentrations of NO_3^- and NO_2^- pre-inoculation in LB and that the concentration of NO_2^- was not affected by NO_3^- (Figures 3B,C).

DYNAMIC INCREASE IN DENITRIFICATION REDUCTASE PROMOTER ACTIVATION AT 400 μM NO_3^-

The reporter strains were grown under anoxia for 20 h supplemented with 400 μM NO_3^- and the fluorescence measured post chloramphenicol addition with OFR by flow cytometry (Figure 4). The activity of all four promoters was significantly increased during supplementation with 400 μM NO_3^- , but the time course varied. The Nar activity showed a small peak at 2 h ($p < 0.0001$) in comparison to Nor, which showed a stronger peak at 2–3 h ($p < 0.0001$). Nir and Nos first showed significant upregulated promoter activity after 1 h and continued to be so over 20 h ($p < 0.0001$ and 0.0273). In all reductases other than the Nos, there were also periods of significant upregulation in the unsupplemented control, but for shorter periods of time and with lower peaks. The transcripts of the denitrification reductase genes in PAO1 grown under anoxia with 400 μM NO_3^- hours showed significant upregulation of *nirS*, *norC* and *nosR* after 3 h (p -values = 0.014, 0.014, and 0.04) (Figure 5).

We noted that anoxic conditions alone were apparently sufficient to trigger upregulation of some denitrification reductases as previously observed in other planktonic and biofilm studies (Härtig and Zumft, 1999; Hentzer et al., 2005; Schreiber et al., 2007) and that LB is a rich medium which we found to contain $\sim 20 \mu M$ NO_3^- . We speculate that this is an explanation for the observed upregulation in our unsupplemented controls. However, the focus of the present study is on the differences due to the additional physiological levels of NO_3^- supplementation.

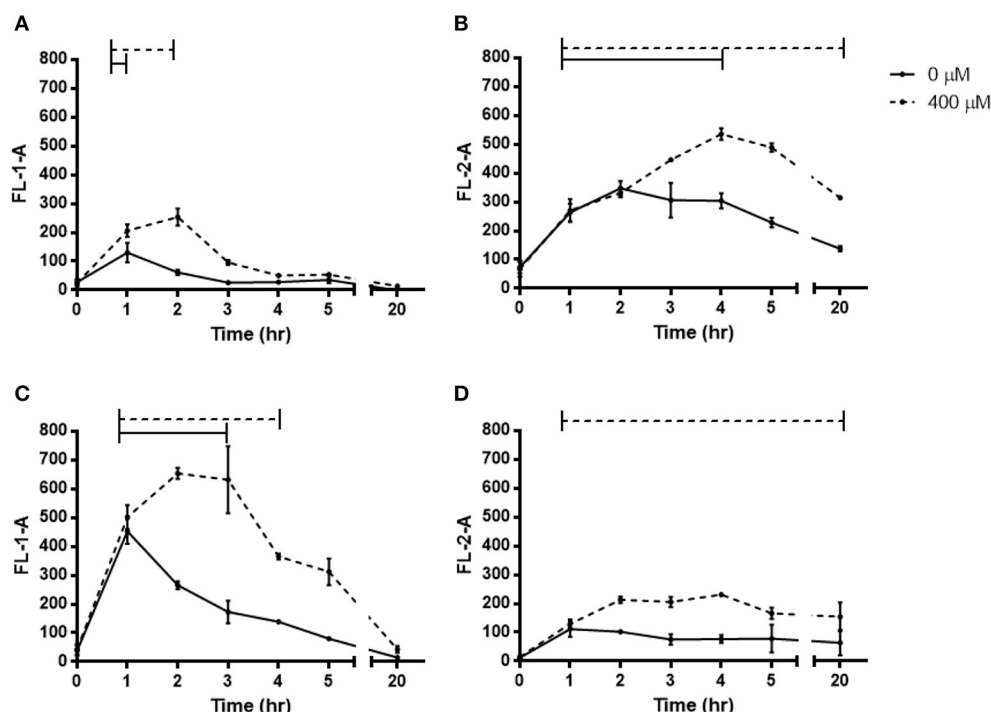


FIGURE 4 | Fluorescence of denitrification reporter promoters of *P. aeruginosa* PAO1 during anoxic growth with 0 or 400 μM NO_3^- . (A) *narK1*-eGFP, (B) *nirS*-dsredexpress, (C) *norC*-eGFP, (D) *nosZ*-dsredexpress.

Mean \pm s.e.m. as measured by flow cytometry from triplicates. eGFP was detected in FL1-A, DsRed-Express was detected in FL2-A. Bars above represent the period of time of significant increase $p \leq 0.05$.

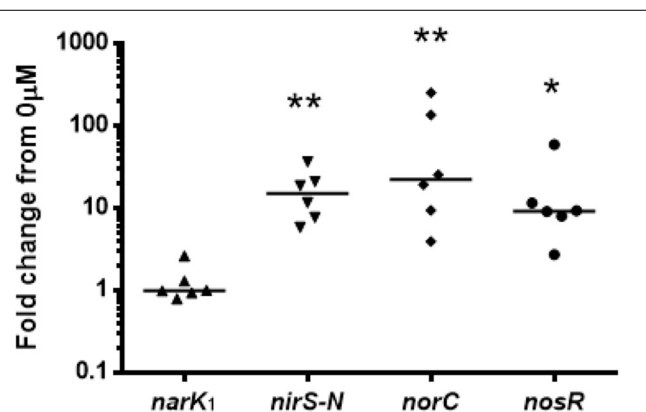


FIGURE 5 | qRT-PCR on denitrification reductase genes of *P. aeruginosa* PAO1 under anoxic growth for 3 h in LB with 0 or 400 μM NO_3^- .

Expression was normalized to the housekeeping gene *rpoD*. Values show fold change from 0 μM with theoretical median of 1. $N = 6$ with median. * $p \leq 0.05$, ** $p \leq 0.01$.

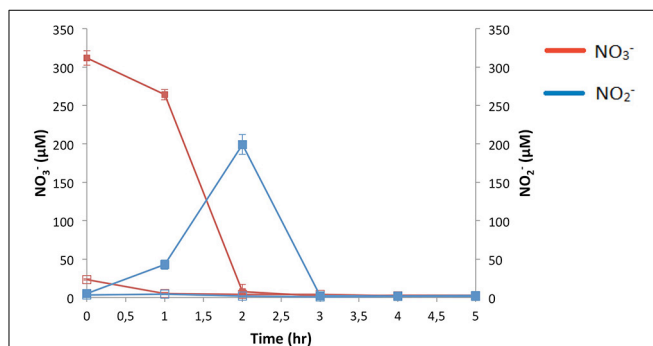


FIGURE 6 | Consumption and production of NO_3^- and NO_2^- during anaerobic growth of *P. aeruginosa* PAO1. $N = 4$, mean \pm s.e.m. Initial NO_3^- : Open squares 0 μM KNO_3 , closed squares 400 μM KNO_3 .

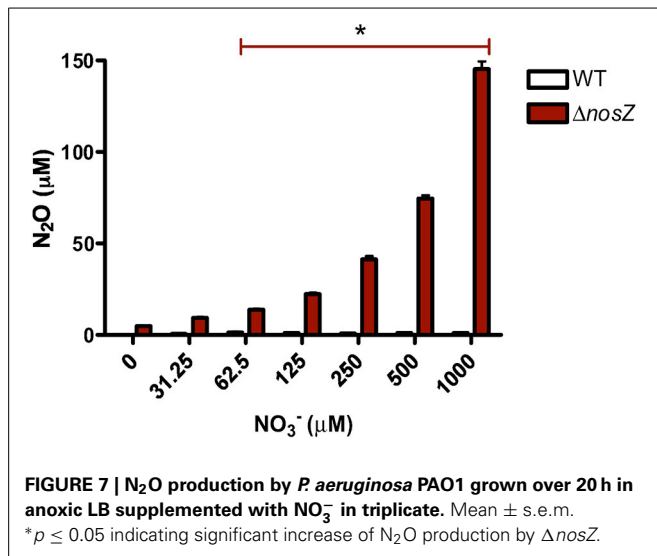
NO_3^- IS RAPIDLY CONSUMED WITH A SUBSEQUENT TRANSIENT INCREASE OF NO_2^- IN ANOXIC GROWTH MEDIA

Medium from the reporter PAO1 strains showed that 400 μM NO_3^- was depleted to below the detection level of the Griess assay within just 2 h with a transient increase in the product of its reduction, NO_2^- (Figure 6). This rapid depletion combined with the time taken to harvest the supernatant is the likely

explanation for the initial time point showing somewhat less than the expected 400 μM NO_3^- , as the media pre-inoculation was tested and confirmed to contain 400 μM NO_3^- .

N_2O IS PRODUCED IN RESPONSE TO NO_3^- SUPPLEMENTATION

The ΔnosZ mutant and a WT PAO1 strain were grown with physiological concentrations of NO_3^- under anoxia and the average N_2O accumulation over 20 h detected. With as little as 62.5 μM NO_3^- , a significant production of N_2O was stimulated ($p = 0.0125$) (Figure 7). The WT produced $<10 \mu\text{M}$ of N_2O at all experimental concentrations of NO_3^- ; confirming



the defective conversion of N₂O to N₂ by the ΔnosZ mutant.

DISCUSSION

P. aeruginosa is able to thrive under anoxic conditions by performing denitrification. Our initial studies performed with clinical isolates and PAO1 under anoxic conditions showed that physiological concentrations of NO₃⁻ are sufficient to support a significant increase in growth rate, even in cultures supplemented with less NO₃⁻ than the 400 μM applied by Palmer et al. (2007).

The anoxic growth rates obtained are comparable to those reported in CF lungs and sputum, except for the very low growth rates in some of the clinical samples from CF lungs and sputum (Yang et al., 2008; Kragh et al., 2014). This is likely due to our planktonic studies representing a slightly faster growth rate as the shaken cells have equal access to nutrients in the media, whereas in the biofilm mode of growth in the lungs, internal cells are assumed to have access to less nutrients, including NO₃⁻, due to diffusion limitation and utilization by cells at the periphery of the biofilm (Bjarnsholt et al., 2009). Additionally, ~10% of the cells in clinical samples appear to be in stationary phase (Yang et al., 2008) whereas the growth rates in our experiments are calculated from the exponential growth phase. In the lungs, *P. aeruginosa* is also under continual attack from the host inflammatory response and antibiotic stress (Høiby, 2006), which is also likely to reduce the rate of growth.

Nar is required for anoxic growth at physiological levels of NO₃⁻ in CF sputum (Palmer et al., 2007) and accordingly NO₃⁻ reduction alone is able to generate some growth as seen in the ΔnirS-N mutant, unable to perform the subsequent NO₂⁻ reduction and therefore blocking denitrification. However, we demonstrate that the ΔnirS-N mutant requires the highest physiological concentration of 1 mM NO₃⁻, seen only in exacerbated CF patients following antimicrobial treatment (Grasemann et al., 1998), to enable a significant increase in growth rate as well as a significant reduction of growth as compared to the WT. This indicates a substantial additional contribution of denitrification

to the observed anoxic growth of *P. aeruginosa* at physiological levels of NO₃⁻. Anoxic growth of mutants with defective Nir was also reduced at concentrations of NO₃⁻ greater than physiological levels [10 mM (Schreiber et al., 2007) and 100 mM (Filiatrault et al., 2006)].

In addition to supporting anoxic growth, Nir may promote the persistence of *P. aeruginosa* in the infected CF lungs as Nir is required for type III secretion resulting in prolonged survival in human monocytes (Van Alst et al., 2009) and enhanced virulence (Filiatrault et al., 2006). This supports the idea that denitrification is necessary for achieving the observed growth of *P. aeruginosa* in the lungs.

The dynamic increase in the promoter activity of all four denitrification reductases over 20 h and increased expression of all transcripts but the *narK1* at 3 h with 400 μM NO₃⁻ further emphasized the engagement of denitrification. The explanation for the transient peak in Nar expression and lack of upregulation at 3 h became apparent after analysing the growth media for NO₃⁻ content, showing depletion after just 2 h. Expression of Nar is controlled in part by the two-component NO₃⁻-sensing regulator NarX-L (Schreiber et al., 2007; Schobert and Jahn, 2010) so presumably after NO₃⁻ depletion Nar expression is no longer induced. The Nor is also proposed to be under NarX-L control (Schobert and Jahn, 2010), which would explain the decrease in promoter activity after 2 h.

The Nar product, NO₂⁻ displayed a transient increase in the growth media which was similarly rapidly depleted by 3 h. However, the expression of Nir and Nos appeared to be less tightly regulated by N-oxide concentration as they were still significantly upregulated at 20 h, and in the case of the Nir, only a modest increase in expression was stimulated by NO₃⁻ supplementation. The additional O₂-sensing Anr regulator and downstream NO sensing dissimilative nitrate respiration (Dnr) regulator (Schreiber et al., 2007; Schobert and Jahn, 2010; Trunk et al., 2010; Arai, 2011) may play a more important role in Nir and Nos regulation (Schreiber et al., 2007; Schobert and Jahn, 2010) and hence their expression more responsive to the anoxic conditions and the downstream denitrification product NO resulting in prolonged increase in expression.

As N-oxides are not exclusively utilized in strict denitrification—NO₃⁻ reduction is also able to feed into dissimilatory pathways for ammonia excretion or into assimilatory pathways for incorporation into biomolecules (Zumft, 1997)—we also studied N₂O production. The known pathways for N₂O production are denitrification and nitrification via oxidation of hydroxylamine to N₂O (Khalila et al., 2004). As of yet, there is no evidence to suggest that *P. aeruginosa* performs or possesses homologs of the enzymes required for nitrification [PAO1 Genome Sequence, Pseudomonas Genome Database (Winsor et al., 2011)]. Additionally, the process requires O₂ (Meyer et al., 2008) and thus for our anoxic experiments we assumed that all detected N₂O was produced via the denitrification pathway and therefore acted as a specific marker. In the *P. aeruginosa* nitrous oxide reductase mutant ΔnosZ, unable to reduce N₂O to N₂, we found a significant accumulation of N₂O in the anoxic growth media with as little NO₃⁻ supplementation as 62.5 μM. This finding further supports that *P. aeruginosa* employs denitrification to

obtain energy for growth at physiological levels of NO_3^- in anoxic conditions.

Interestingly, one late clinical strain isolated from a patient chronically infected for 35 years, was unable to grow in anoxic conditions with NO_3^- . This may relate to adaptations of more flexible nitrate metabolism over time, with denitrification suggested to no longer be essential for growth for some strains at later phases of infection (Oberhardt et al., 2010; Cramer et al., 2011). While NO_3^- was unable to stimulate anoxic growth of the late isolate, alternative anoxic growth may occur by arginine fermentation (Vander Wauven et al., 1984) and survival could be facilitated by pyruvate fermentation (Eschbach et al., 2004) and phenazine redox cycling (Glasser et al., 2014; Quinn et al., 2014). Alternatively, the late isolate may have adapted to favor growth in the alveoles where oxygen is supplied by inspired air and capillaries surrounding the alveoles (West, 2000).

It has previously been shown that micro-oxic conditions are able to support growth of *P. aeruginosa* (Alvarez-Ortega and Harwood, 2007). Here we substantiate that physiological concentrations of NO_3^- are also able to support growth by denitrification in anoxic conditions, highlighting the flexibility of *P. aeruginosa*'s ability to survive in a range of environments in the CF airways (Worlitzsch et al., 2002; Aanaes et al., 2011; Kolpen et al., 2014a). Interestingly, physiological concentrations of NO_3^- may also enhance growth of *P. aeruginosa* even at micro-oxic conditions since a growth advantage of laboratory strains carrying a *lasR* mutation, which is associated with severe lung damage in infected CF patients (Hoffman et al., 2009), was obtained with supplementation of only 125 μM NO_3^- in conditions with limited O_2 mass-transfer (Hoffman et al., 2010).

In conclusion, *P. aeruginosa* is readily able to adapt to the physiological levels of NO_3^- and lack of O_2 in the infected CF lungs by employing denitrification. The ability of *P. aeruginosa* to grow under anoxia has clinical relevance as multiple classes of antibiotics are known to have reduced efficacy in the absence of O_2 (Walters et al., 2003; Dwyer et al., 2014). Denitrification may consequently be a major factor in *P. aeruginosa*'s observed tolerance to antibiotic treatment in CF patients (Høiby et al., 2010). This tolerance may be overcome by directing antibacterial treatment against denitrification as suggested by the downregulation of *narG* and *narH* resulting in increased susceptibility of *P. aeruginosa* to fosfomycin and tobramycin combination treatment under anoxic conditions (McCaughey et al., 2013). Denitrification has also recently been proposed to contribute to the pathogenicity of bacterial species infecting CF lungs due to its activity in *P. aeruginosa* as well as the highly pathogenic species *Achromobacter xylosoxidans* and *Burkholderia multivorans* in comparison to the less pathogenic *Stenotrophomonas maltophilia* which is unable to perform denitrification (Kolpen et al., 2014c). Accordingly, our results underline the importance of developing novel treatments that target *P. aeruginosa*'s flexible metabolism more broadly, with the denitrification reductases presenting potential targets, and that can be administered to reach the varied lung microenvironments that *P. aeruginosa* is able to adapt to and persist within.

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